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Total quantification and extraction of shikimic acid from star anise (*Illicium verum*) using solid-state NMR and cellulose-dissolving aqueous hydroxide solutions

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ABSTRACT

Many pharmaceutically relevant molecules are still extracted directly from lignocellulosic biomass sources. As this can be a bottleneck in supply, total quantification followed by total extraction are desirable processes to ensure as much as possible is obtained, at the optimal time in the growth cycle. Herein we report the application of solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy to quantify shikimic acid present inside Chinese star anise (or star aniseed, *llicium verum*). Subsequently, methanol soxhlet extraction is compared with conventional aqueous hydroxides (*i.e.* sodium hydroxide) and cellulose-dissolving aqueous hydroxides (*i.e.* tetraethylammonium and tetrabutylammonium hydroxide) for shikimic acid isolation. Methanol extraction isolated *ca.* 6.6 ± 0.1 weight percent (wt %) shikimic acid (post-purification), and solid-state NMR confirmed extraction was incomplete even after 72 hours. Conversely, dissolution of the star anise at room temperature in tetrabutylammonium hydroxide ($[N_{4444}]OH$) allowed isolation of *ca.* 14.0 ± 0.6 wt % shikimic acid (post-purification). Solid state NMR confirmed the star anise initially contained 19 ± 3 wt % shikimic acid, which was quantitatively extracted after dissolution in the aqueous hydroxide solution. The solubility of the star anise and the kinetics of the shikimic acid extraction were also briefly evaluated.

KEYWORDS

Lignocellulosic biomass; biomass pre-treatment; star aniseed (*llicium verum*); solid-state NMR; extraction; isolation

1. INTRODUCTION

Many precursors of medicinal compounds are commercially extracted from biomass, including virtually all of the widespread medicinal opiates.[1] The insufficient availability of certain drugs has been directly related to an insufficient quantity of the required biomass at the time.[2-5] Despite efforts in the areas of total synthesis and genetically engineered biochemical routes,[2, 6] the complexity of many pharmaceutically relevant molecules and the unrivalled convenience and economics of well-established biomass means this situation is likely to persist for a considerable period of time to come.

The quantities of pharmaceutically relevant molecules present in a specific species are known to vary as a function of season and growth conditions.[4] Chemical processes are then required to isolate and purify the specific molecule(s) from the rest of the biomass at the most appropriate time. Two major requirements for sustainable processes along these lines are (i) a facile analytical technique for monitoring the biomass to identify when it contains the optimal concentration of desired molecules, and (ii) a total extraction procedure to isolate all of the desired materials, under mild conditions using innocuous reagents.

Shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) is an important precursor in the production of oseltamvir (Tamiflu®), an avian flu drug,[6] and other anti-biotics.[2] Shikimic acid is produced biologically via the shikimate pathway; it is the precursor to aromatic amino acids[7] and other plant-produced secondary products such as salicylic acid, tannins, anthocyanins, coumarins, *etc.*[8] It also has its own possible functions in plants, such as metal detoxification and phosphorous mobilisation.[9] Due to the widespread importance of shikimic acid for biomass such as plants, bacteria, fungi and parasites, but absence in mammals,[2] some commercial herbicides target the shikimic acid pathway.[8]

The total synthesis of shikimic acid was first achieved in the 1960's, but is still challenging enough that it is not economically viable.[6] Most shikimic acid utilised by the pharmaceutical industry are obtained as plant extracts and, increasingly, from metabolically engineered bacterial strains.[2]

Shikimic acid has been extracted from traditional medicinal plants such as *Belamcanda chinensis*,[10] *Dendrobium huoshanese,*[11] *Hypericum laricifolium*[12] and *Pteridium aquilinum*.[13] However, it is also found in aquatic weeds,[7] wheat plants,[14] peaches,[15] honey,[16] coconut water,[17] a wide variety of berries,[18-22] grapes[23, 24] and wine[25-27]. Shikimic acid is also common in trees, and has been isolated from the bark,[28] leaves,[29] needles,[30, 31] roots[32] and seeds[33] of Sweetgum (*Liquidambar styraciflua*) and Pine (*Pinus*) varieties.

The highest yielding plants for shikimic acid are those of the *Illicium* variety, with some containing almost 25 wt % shikimic acid in their dried fruits.[34] However, the majority of the *Illicium* species are also poisonous.[6] A major exception is fruit of the Chinese star anise (or star aniseed, *Illicium verum*). These are widely employed in cooking,[35] and up to *ca.* 17 wt % and *ca.* 10 wt % shikimic acid have detected and physically isolated, respectively.[4, 36]

Quantification of shikimic acid by extraction – without its direct isolation – has been extensively reported. For example, Cai *et al.* investigated the aqueous extraction of shikimic acid from pulverised star anise assisted by ultrasonic and microwave irradiation, followed by UV analysis of the extract; up to 1.4 wt % and 2.8 wt % shikimic acid was quantified, respectively.[37] Liu *et al.* reported shikimic acid contents for star anise approaching 15 wt %, by ultrasound-assisted extraction from macerated samples in 90:10 methanol:water, followed by trimethylsilanisation and GC-MS quantification.[4] Avula *et al.* sonicated

ground star anise in methanol for 20 minutes, using three successive extractions, followed by HPLC-MS and HPLC-UV quantification; up to 17.77 ± 0.026 wt % shikimic acid was found.[34]

The physical isolation of shikimic acid from star anise has also been reported. Adams *et al.* reported the isolation of *ca.* 6.5 wt % shikimic acid from star anise, after ethanol-soxhlet extraction and purification.[38] Xue *et al.* used a 5 h ethanol-soxhlet extraction, followed by ethyl acetate washing and flash column chromatography through a high-capacity molecularly-imprinted polymer column, to recover an unreported quantity of >95% purity shikimic acid (based upon mass-spectroscopy).[39] Ohira *et al.* claimed 100% (quantitative) extraction of *ca.* 8 wt % shikimic acid from star anise within 4 minutes using water at 150 °C and 15 MPa;[5] however, this quantitative extraction assignment appears to be based upon the assumption that a 48 h methanol-soxhlet extraction is almost quantitative, and the highest water-extraction value (8 wt %) was therefore arbitrarily assigned as 100% quantitative extraction. Recently, Just *et al.* employed a household espresso machine to perform pressurised hot solvent extraction of shikimic acid from star anise powder, using 30:70 ethanol:water, and isolated up to 5.5 wt % shikimic acid, post-purification.[40]

The above reports have focussed upon solid-liquid extraction. Others have exploited the ability of ionic liquids to act as near-universal, virtually non-derivatising solvents for a wide range of biomass[41, 42] and lignocellulosic components,[43, 44] to improve shikimic acid extraction from (solubilised) biomass. Usuki *et al.* used the ionic liquid [Bmim]Cl to dissolve and thus significantly enhance the extraction of shikimic acid from *Ginkgo biloba* leaves (up to 2.3 wt %), and isolate it using an ion exchange resin.[45, 46] Chen *et al.* used water-ionic liquid mixtures and ultrasound-assisted extraction, followed by HPLC analysis, to confirm up to 1.5 wt % shikimic acid could be extracted from pine needles.[47] Zirbs *et al.* achieved complete solubilisation of star anise in the ionic liquid [Emim][OAc] by heating to

100 °C for 10 min under high absorption microwave irradiation, and recovered up to 10.2 ± 0.6 wt % spectroscopically pure shikimic acid using an ion exchange resin.[36] While Zirbs *et al.* were unable to state what proportion of the original shikimic acid had been extracted and recovered, it was highlighted as the highest physically isolated value reported for star anise.[36] The same group demonstrated that star anise can be dissolved in ionic liquids, and the solubilised shikimic acid derivatised to form relevant pharmaceutical intermediates in a 'reactive dissolution' one-pot approach.[48]

Plant-derived biomass is almost exclusively lignocellulosic in nature, being composed of primarily cellulose, hemicellulose and lignin.[44] Due to the recalcitrance of such biomass, solid-state ^{13}C-Nuclear Magnetic Resonance (NMR) spectroscopy has been extensively employed to investigate the chemical nature and composition of these materials in pristine and modified lignocellulosic biomass.[49] Although exotic materials such as ionic liquids have been widely employed to dissolve whole biomass, concentrated aqueous hydroxide solutions with certain bulky organic cations have been known to dissolve cellulose since the 1930's.[50] Recently, they have been investigated for their interactions with whole lignocellulosic biomass. [51-54] Briefly, the lignocellulosic biomass structure can be significantly disrupted and even fully solubilised, significantly facilitating downstream processing such as acid hydrolysis or enzymatic digestion.[51]

This article first details an investigation of whole star anise powder, using solid-state Nuclear Magnetic Resonance (NMR) spectroscopy to definitively quantitate the shikimic acid content present in the whole biomass sample. Subsequently, a range of hydroxide salts are investigated as shikimate extraction media; it is demonstrated that aqueous tetrabutylammonium hydroxide both completely dissolves the star anise at room temperature and all of the shikimic acid is removed from the star anise. Finally, the shikimic acid is recovered from solution. The key emphasis here is not the shikimic acid, but rather the

demonstration that (i) solid-state NMR can be employed for total quantification of pharmaceutically-relevant molecules *inside* lignocellulosic biomass, and (ii) a predominately aqueous solution can be employed under ambient conditions to fully solubilise – and thus fully extract – these pharmaceutically relevant molecules. Development of step (i) allowed independent confirmation of the success of step (ii).

2. Materials and Methods

All hazardous materials were kept from the environment and effectively handled following UNSW guidelines (document HS321), and following the plans and policies outlined in UNSW's Green Lab Environmental Compliance Program. Unless otherwise noted, all commercial regents were purchased from Sigma-Aldrich (Castle Hill, Australia).

2.1 Soxhlet treatment of star anise and crude shikimic acid isolation

Ground Star anise (5 g, Macrotaste™) was placed in a cellulose thimble and extracted with refluxing methanol (250 mL) in a soxhlet for 72 hours. The resulting dark brown methanol extract was evaporated to dryness *in vacuo* to leave a dark tar and a layer of green oil. The mixture was gently washed with hexane, and the oil/hexane mixture was discarded. The tarry component was then taken up into water (100 mL) and heated to 80 °C, where upon 37-40 v/v % aqueous formaldehyde (1 mL) was then added to the mixture and boiled for 5 minutes. The resulting brown precipitate was removed by filtration, and the filtrate (a clear amber solution) was then subjected to further ion-exchange column purification as described below, to yield a light yellow solid.

2.2 Treatment of star anise with hydroxide solutions

Ground star anise (2.5 g) was stirred at room temperature in 25 mL of sodium or tetraalkylammonium hydroxide solutions; these solutions consisted of 25 water molecules per hydroxide ion pair. After 48 h the brown or black solutions were filtered through a 0.22 μm Millipore membrane in order to remove any undissolved material. The filtrate was then collected and subjected to ion-exchange purification, as described below, to yield orange solids as the crude product. Some of the $[N_{4444}]OH{\bullet}25H_2O$-isolated crude (0.0472 g) was further purified by dissolving in water (5 mL) and washed with dichloromethane (3 x 5 mL). It was then dried at 110 °C overnight to yield a light yellow solid (0.0286 g, 61 % yield). This was confirmed to be shikimic acid by ^{1}H NMR (D_2O, ppm); 6.73 (C=C<u>H</u>, 1H, d); 4.26 (C=CH-C<u>H</u>OH, 1H, t); 3.94 ($CH_2C\underline{H}OH$, 1H, dt); 3.68 (CHOH-C<u>H</u>OH-CHOH, dd); 2.64 ($C\underline{H}^aH^b$, 1H, dd); 2.13 ($CH^a\underline{H}^b$, 1H, dd).

2.3 Ion-exchange purification and isolation of shikimic acid-extracts

Amberlite 400 exchange resin in chloride form (50 mL) was exchanged into its acetate form by treatment with sodium acetate (1 M) on a column until there was no visible trace of precipitate when the eluent was treated with aqueous silver nitrate (0.1 M). The resins were then washed with methanol to remove any organic material. The extracts from soxhlet treatment (section 2.1) and hydroxide treatment (section 2.2) were passed through the anion exchange column and washed with water until the eluted water was clear. The immobilised shikimic acid was then eluted with an aqueous acetic acid solution (25 v/v %) and monitored with TLC. The eluted shikimic acid could then be isolated by drying the solution *in vacuo*.

2.4 Qualitative $^{13}C\{^1H\}$ cross-polarisation solid-state NMR. The ^{13}C NMR experiments were carried out on Bruker AVANCE III 300 spectrometer with a 7 Tesla superconducting magnet operating at frequencies of 300 MHz and 75 MHz for the ^1H and ^{13}C nuclei respectively. The samples were finely ground and *ca.* 80 mg sample was center-packed into 4 mm zirconia rotors with Kel-f® caps. The ^{13}C CPMAS experiments were acquired at 8 kHz MAS with a 2 ms cross polarisation contact time and 2 s recycle delay. The pure shikimic acid demonstrated very long ^1H-T_1 relaxation times, hence the recycle delay was set to 60 s to ensure sufficient equilibration of the magnetization.

2.5 Quantification of the shikimic acid content by solid-state ^{13}C direct polarisation NMR. Quantitative ^{13}C NMR spectra were measured with spinning at 12 kHz MAS using a direct polarisation hahn-echo sequence and recycle delays of 100 s which were found to be sufficient to recover > 95 % of the ^{13}C magnetization as determined by a ^{13}C-CP-T_1filter[55] experiment. ^{13}C-90° and ^1H-90° pulse lengths of 4 μs and 3.2 μs respectively were used along with ^1H SPINAL64 decoupling at field strength of 80 kHz during acquisition. A total of 2048 signal transients were co-added to provide sufficient signal-to-noise for the direct polarisation spectra. This corresponded to 48 h of measurement. Adamantane ($C_{10}H_{16}$) was used as an external spin counting-reference to quantify the amount of shikimic acid in the star anise sample; the actual spectra as shown in Figure 2 in the Results and Discussion section. ^{13}C pulse-length experiments confirmed that possible differences in the internal B1 field homogeneities between the two samples were negligible, with a difference in the adamantane/star anise intensity by only 1.002.

An example of the procedure used to quantify the wt % of shikimic acid present in star anise is:

a) 51.2 mg of adamantane and 85.6 mg of star anise (which occupy approximately the same volume) were packed into 4 mm zirconia rotors. Similar sample volumes were used to account for the variability in the response of the NMR coil (the radio-frequency field inhomogeneity) at different points along the rotor axis.

b) The integrated area of the quantative-^{13}C NMR signal of adamantane was measured and multiplied by a factor of 1.672 (*i.e.* 85.65 mg/51.2 mg) to account for the difference in the mass of the samples, and set to 100.

c) 85.65 mg of adamantane, which has a molar mass of 136.24 g·mol^{-1}, contains 75.59 mg of carbon atoms, which now correspond to a ^{13}C NMR signal integral of 100.

d) The integrated area of the ^{13}C NMR signal integral of the COO peak for shikimic acid in the star anise was also measured. Each 12.01 g of COO carbons quantified in the sample equate to 174.15 g of shikimic acid.

Following this procedure, the COO peak for shikimic acid in the star anise measured relative to the adamantane was found to be 1.34; this corresponds to 1.01 mg of carbon atoms present as COO in the 85.6 mg star anise sample. Thus the sample contained 14.66 mg of shikimic acid, or 17.1 wt %.

The primary source of error was investigated. The signal to noise ratio for the integrated COO peaks was typically *ca.* 13.7; this equates to *ca.* 7% error. Measuring the same sample in duplicate found values of 19.2 wt% and 19.5 wt% shikimic acid, corresponding to an error of less than 1 %. Three samples were separately packed and measured against adamantane in order to have triplicate measurements that probed the reproducibility; this found 17.1 wt %, 19.35 wt % and 20.1 wt %, corresponding to a packing/reproducibility-error of *ca.* 8% error. Therefore packing reproducibility and background noise were the two major sources of error; combining the values and errors yields 18.9 ± 1.8 wt %.

3. RESULTS AND DISCUSSION

3.1 Solid-state NMR analysis of the star anise. Many reports have described the extraction of shikimic acid from star anise,[4, 5, 34, 36, 37, 39, 40, 48, 56] and in some cases have used extraction to 'quantify' the total shikimic acid content in the star anise.[4, 5, 34] These 'quantified' values assume 100% extraction and range widely between 8 wt % and 17 wt %.[4, 5, 34] Arguably, the shikimic acid present in star anise has only ever been quantified as 'extracted star anise'. As such, the shikimic acid content of the as-received, untreated star anise powder was first definitively and independently quantified by solid-state NMR of the whole biomass.

Solid-state ^{13}C NMR of pure shikimic acid was first obtained (Figure 1(a)), which highlighted the relevant carbon atoms present in the shikimic acid molecule. Solid-state NMR of the star anise powder (Figure 1(b)) demonstrated multiple features, consistent with shikimic acid and lignocellulosic biomass.

Comparison of the shikimic acid features in Figure 1(a) with those of star anise (Figure 1(b)) and star anise where shikimic acid had been quantitatively extracted (*vide infra*) highlighted that only the COO signal of the shikimic acid at 172 ppm did not significantly overlap with the lignocellulosic features. The COO signal for pure crystalline shikimic acid had a full width at half maximum (FWHM) of 0.9 ppm, while the COO signal in the star anise had a FWHM of 6 ppm, indicating the amorphous structure of shikimic acid in the star anise, and would therefore preclude X-ray diffraction based quantification. The COO peak in star anise is also more complex than a single feature, due to the diverse and heterogeneous environments found in the biomass compared to crystalline shikimic acid powder. It should be noted that this assignment assumes that the shikimic acid content is far in excess of any

other COO present in the biomass, such as glucuronic acid. The molecular nature of the hemicellulose in star anise is currently unknown, but the glucuronic acid content in hardwood and softwood is typically 1.6 wt % or less,[57] *i.e.* at least an order of magnitude lower than reported shikimic acid values.[34] Furthermore, hemicellulose represents an even lower proportion of the whole in star anise, due to the relatively high shikimic acid (up to 17 wt %[34]) and oil (up to 12 wt %[5]) content.

Using the COO signal of the shikimic acid, and adamantane as an external spin-counting reference, the shikimic acid present in the star anise could thus be quantified. This removes the necessity of having a detailed molecular structure of all the components in the star anise. Full quantitative details are included in the Materials and Methods section. Briefly, the ^{13}C solid-state NMR spectra of star anise and adamantane were recorded by direct polarisation for 48 hours, in order to reduce the signal-to-noise to a suitable level. The shikimic acid peak in star anise (Figure 2(a)) was then quantitatively compared with pure adamantane (Figure 2(b)). The direct polarisation method quantitatively detected both solids and liquids present in the samples, as demonstrated by the appearance of the essential oil features in Figure 2(a) at *ca.* 30 ppm (*cf.* their absence in Figure (1(b)). Repeating this experiment in triplicate quantified that 18.9 ± 1.8 wt % shikimic acid is present in the star anise sample (full discussion of process in the experimental, section 2.5). Given the possible presence of small quantities of acid sugars in the hemicellulose content of the star anise, this is more appropriately expressed as 19 ± 3 wt % shikimic acid. This value of 19 ± 3 wt % agrees well with the highest reported shikimic acid content of star anise (17.77 ± 0.026 wt %), which was achieved by three-fold ultrasound-assisted methanol extraction of ground star anise powder, followed by HPLC-MS quantification.[34] It should be noted that to date, only extraction-derived values have been reported as a proxy for content, which have varied between *ca.* 8 and 18 wt % shikimic acid in star anise (*llicium verum)*.[5][34] Values of up to

24.8 wt % shikimic acid have been reported from extraction-based quantification using fruits of the same genus (*Illicium religiosum*).[34]

3.2 Soxhlet extraction of the shikimic acid. The most frequently reported method for the extraction of shikimic acid from star anise is arguably extraction from a ground powder using methanol or ethanol in a soxhlet apparatus.[4, 5, 38, 56] Therefore ground star anise was placed in a cellulose thimble and extracted in a soxhlet apparatus with refluxing methanol (5 g powder for 250 mL methanol) for 72 hours. The resulting dark brown extract was evaporated to dryness *in vacuo* to give a tarry component and a layer of thick green oil (mostly anethole[5]). This was subjected to further purification, with full details in the Materials and Methods section.

Briefly, literature precedent was followed and so a hexane wash was used to remove the oil, and the remaining tarry component was diluted with water and boiled with formaldehyde[38, 56] to remove proteins as a brown precipitate. The resulting clear shikimic acid-rich amber solution was dried, and *ca.* 10.8 wt % of the initial mass was isolated as a shikimic acid-rich crude phase. This crude was then subjected to ion exchange purification[38, 56] by loading upon a hydroxide-loaded anion exchange membrane, washing, and then eluting the retained shikimate salt with acetic acid. This resulted in pure shikimic acid (6.6 ± 0.1 wt % of the initial star anise powder, in duplicate experiments); identification and purity was assessed by NMR (confirming purity of the organic components) and TGA (left no ash, confirming no inorganic contaminants). The obtained crude value (10.8 wt %) and purified value (6.6 wt %) agrees well with the literature values previously reported where this technique was employed to physically isolate the final extract (*cf.* 5-8 wt % shikimic acid from star anise).[36, 38, 56]

A recovery of only 6.6 ± 0.1 wt % shikimic acid, relative to the quantity determined by solid-state NMR, implies that some material likely remains in the star anise powder, even after 72 hours of extraction (and despite assumptions by others that soxhlet extraction with alcohols is quantitative[4, 5, 34]). The post-extraction star anise in the thimble was therefore investigated by solid-state NMR. Figure 1(c) displays the resulting ssNMR, which clearly indicates the presence of residual shikimic acid (in the 160-180 ppm), demonstrating incomplete removal. The features in the 160-180 ppm region did not decrease significantly relative to the initial star anise sample, hence it is important to note that the cross-polarisation technique used is not quantitative, and the extensive oil content was likely quantitatively extracted, thus making the residual shikimic acid actually appear relatively more concentrated in the post-extraction sample.

3.3 Solubility of star anise in partially hydrated alkali systems. Various aqueous tetraalkylammonium and tetraalkylphosphonium hydroxides can dissolve cellulose and even whole lignocellulosic biomass,[51-54] at room temperature and despite being predominately water.[51] We have recently demonstrated that for a fixed [cation]OH:H_2O ratio of 25:1, the cellulose solubility increases as the alkyl chain length increases, *e.g.* from *ca.* 0 wt % cellulose for tetramethyl- ($[N_{1111}]^+$), through to *ca.* 5 wt % cellulose for tetraethyl- ($[N_{2222}]^+$) and *ca.* 9 wt % cellulose for tetrabutylammonium ($[N_{4444}]^+$) hydroxide.[58] Cellulose was virtually insoluble in longer chain analogues, such as tetrahexylammonium hydroxide.[58] Lignin and silica are also highly soluble in all of these media.[51] Therefore the solubility of star anise powder was investigated in a range of hydroxide systems with a fixed [cation]OH:H_2O ratio of 25:1.

This was initially tested by stirring 0.25 wt % star anise in methanol, NaOH•25H_2O, [N_{1111}]OH•25H_2O, [N_{2222}]OH•25H_2O and [N_{4444}]OH•25H_2O, for 18 h at room temperature. UV-Vis analysis (Figure 3(a)) demonstrated that methanol extracted only some UV absorbing substances, with a small peak present at *ca.* 265 nm; the absorbance increased from NaOH•25H_2O to [N_{4444}]OH•25H_2O, as more material was extracted or dissolved, as plotted in Figure 3(b). The [N_{1111}]OH•25H_2O, [N_{2222}]OH•25H_2O and [N_{4444}]OH•25H_2O demonstrated complete solubilisation of the star anise.

In order to investigate the dissolution and extraction kinetics, 0.25 wt % star anise was stirred in [N_{4444}]OH•25H_2O, and the UV-Vis spectra recorded periodically. The absorbance at virtually all wavelengths increased rapidly before gradually reaching a plateau by *ca.* 48 h; Figure 3(c) arbitrarily plots the absorbance at 265 nm against time. The resulting trend could be fit to a semi-log line fitting, *i.e.* absorbance was linear between 0 and 48 h *vs* the log of time. Extraction (of the UV absorbing components) was *ca.* 50% complete after 1 h, *ca.* 95% complete after 24 h, and complete after *ca.* 48 h; this process could likely be accelerated at higher temperatures.

The total solubility of star anise was also evaluated. Star anise was found to be essentially insoluble in methanol and NaOH•25H_2O, partially soluble in [N_{1111}]OH•25H_2O (up to 2.25 ± 0.25 wt %) while [N_{2222}]OH•25H_2O could dissolve up to 3.75 ± 0.25 wt %. The solubility of star anise in [N_{4444}]OH•25H_2O could not be conclusively determined, as solution became a gel if agitation was ceased after more than 1 wt % star anise was dissolved, and gelation always occurred before true saturation could be achieved. However, more star anise (> 4 wt %) could be dissolved in [N_{4444}]OH•25H_2O than the other samples. Again, these values refer to ambient temperature and could potentially be enhanced by elevated temperatures.

3.4 Extraction of shikimic acid from star anise using partially hydrated alkali systems. In order to investigate shikimic acid extraction and isolation, a deliberately heavy loading of 10wt % star anise in the sodium and tetraalkylammonium hydroxide•$25H_2O$ solutions was employed. These were stirred for 48 h at room temperature; this duration was selected based upon Figure 3(c), which indicates complete dissolution after 48 h. Following this trend, *ca.* 50% dissolution occurs after less than 1 h, *ca.* 80% after 9 h, and *ca.* 100% after 48 h. A photograph of the resulting solutions after stirring for 48 h (in comparison to water and methanol) is shown in Figure 4; this clearly demonstrates the significantly enhanced dissolution and thus extraction in the tetraalkylammonium hydroxide solutions. Afterwards, in order to precipitate the dissolved material an excess of methanol was added, and the resulting solid was removed by filtration. The shikimic acid-rich filtrate was then passed through an ion exchange column (as described in the Materials and Methods section) in order to isolate the shikimic acid; the hexane wash was omitted as no clear oil phase was observed, and the formaldehyde treatment omitted in the interest of sustainability and green chemistry principles. On the anion exchange column the sodium and tetraalkylammonium cations were initially eluted, and can in theory be recycled. The crude shikimic acid was eluted from the column by passing acetic acid, and Table 1 displays the yields for the obtained shikimic acid-rich crude. Increasing amounts of crude were recovered as the cation size increased, corresponding to increased star anise solubility.

Analysis of the shikimic acid-rich crude displayed no inorganic impurities (no ash by TGA) but did display numerous minor impurities by ^{1}H NMR. Notably, unlike the methanol extract there was no distinct oil phase, and it is likely that different impurities were introduced by using the tetraalkylammonium hydroxide solutions. Furthermore, the purification techniques that were found to be effective for the soxhlet-methanol extraction

were not effective for the alkali-extracted samples. Recently, Just *et al.* performed the pressurised hot water-ethanol extraction of shikimic acid from star anise, followed by three successive dichloromethane washes to yield 5.5 wt % 'sufficiently pure by NMR' shikimic acid.[40] By dissolving the crude in D_2O and performing a similar washing step, the impurity peaks for the $[N_{4444}]OH•25H_2O$-isolated shikimic acid were improved such that it was pure by NMR (*i.e.* >95 % purity). A variety of other 'greener' solvents were also investigated, such as hexane, diethylether and ethyl acetate; these were ineffective, and our search for a viable replacement solvent is on-going. Ultimately, the isolated shikimic acid yield corresponded to 14.0 ± 0.6 wt % shikimic acid from the original star anise. This compares well with the independently determined solid-state NMR value of 19 ± 3 wt %; the loss of some material during handling and purification is inevitable on the relatively small scale practised here.

Precipitation of the star anise following $[N_{4444}]OH•25H_2O$ treatment resulted in both the shikimic acid-rich filtrate and a recovered solid; the solid represents the remainder of the star anise. In order to ensure that the shikimic acid had been completely extracted from the star anise, solid-state NMR spectra were recorded for this solid. As displayed in Figure 1(d), the lignocellulosic features were recovered by precipitation yet all features associated with shikimic acid are absent, demonstrating the complete removal of shikimic acid from the recovered solid phase. Some residual $[N_{4444}]^+$ cations can also be observed in the 10-30 ppm region.

Solution-state NMR was also attempted, *e.g.* the star anise was dissolved in $[N_{4444}]OH•25H_2O$, and ^{1}H-NMR attempted, with d_6-DMSO present in an internal capillary. Unfortunately small quantities of star anise resulted in extremely low signals for shikimic acid (relative to the $[N_{4444}]^+$ protons), whereas larger quantities resulted in viscosity, gelation and shimming issues in the tube. As such, solution-state NMR could not be used where solid-

state NMR could. Improving the solvating ability of the system (*e.g.* more dissolved, with a lower viscosity increase) is required for *in situ* solution-state monitoring. It should also be noted that solution-state NMR would still only observe solution-state shikimic acid, and material incorporated in undissolved or biopolymer-rich phases in the solution would not be detected. Therefore successful solution-state NMR would still only represent a complementary technique rather than replacement for solid-state NMR in demonstrating total extraction.

4. Conclusions

By combining solid-state NMR analysis and biomass-dissolving extraction media, we have demonstrated that total quantification and total extraction of pharmaceutically relevant molecules from whole lignocellulosic biomass can be achieved. We have specifically demonstrated the analysis of shikimic acid in star anise using quantitative direct polarisation solid-state ^{13}C NMR. In this study, a total of 19 ± 3 wt % shikimic acid was found to be present in the star anise.

Qualitative solid-state $^{13}C\{^{1}H\}$ cross- polarisation NMR was also employed, in order to investigate the effectiveness of two shikimic acid extraction techniques. Shikimic acid extraction from the star anise using boiling methanol in a soxhlet extraction apparatus, followed by purification of the crude, resulted in the isolation of only 6.6 wt % pure shikimic acid relative to the initial star anise mass. Solid-state NMR confirmed that this extraction technique was incomplete even after 72 hours.

The use of aqueous sodium hydroxide (after 48 h at room temperature) was even less effective than methanol extraction. Conversely, the use of cellulose-dissolving aqueous tetrabutylammonium hydroxide allowed the isolation of 14.0 ± 0.6 wt % shikimic acid, post-purification. Solid-state NMR confirmed that dissolution of the star anise resulted in quantitative shikimic acid extraction. The kinetics of extraction from low loadings of star anise powder was relatively slow (*ca.* 48 hours to completion), although this was achieved at ambient temperature and pressure.

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7. Figures and Tables

Table 1: *Values of the crude shikimic acid-rich solid, isolated post-ionic exchange column treatment, and purified shikimic acid content (pure by NMR and TGA). The wt % values are reported relative to the initial star anise weight, and uncertainties come from duplicate runs.*

Extraction media	Crude shikimic acid-rich solid / wt %	Purified shikimic acid / wt %
Methanol (soxhlet)	10.8 ± 0.1	6.6 ± 0.1
$NaOH•25H_2O$	8.8 ± 0.1	-
$[N_{1111}]OH•25H_2O$	11.5 ± 0.3	-
$[N_{2222}]OH•25H_2O$	14.6 ± 0.5	-
$[N_{4444}]OH•25H_2O$	24.2 ± 1.0	14.0 ± 0.6

Figure 1. *$^{13}C[^{1}H]$ cross-polarisation solid-state NMR of (a) pure crystalline shikimic acid powder, (b) star anise powder, (c) post-methanol soxhlet extraction star anise powder, (d) post-$[N_{4444}]OH$ extraction star anise powder. The region between 160-180 ppm has been expanded in the inset to better visualise the differences in the carboxylate signal of the shikimic acid in the different materials.*

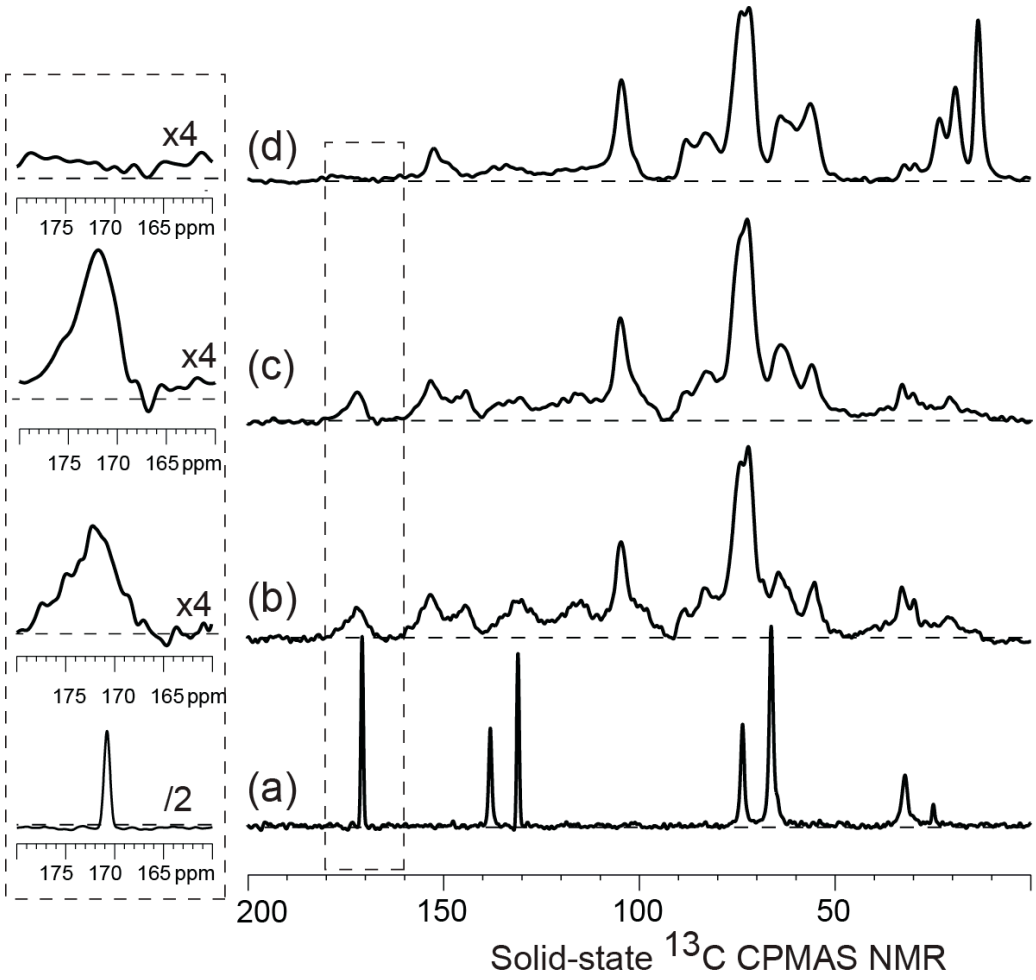


Figure 2. *Quantitative ^{13}C solid-state NMR of (a) adamantane and (b) star anise acquired by direct polarisation. The dominant feature in the star anise at ca. 30 ppm corresponds to the essential oil content. The region between 160-190 ppm has been expanded in the inset to better visualise the differences in the carboxylate signal of the shikimic acid.*

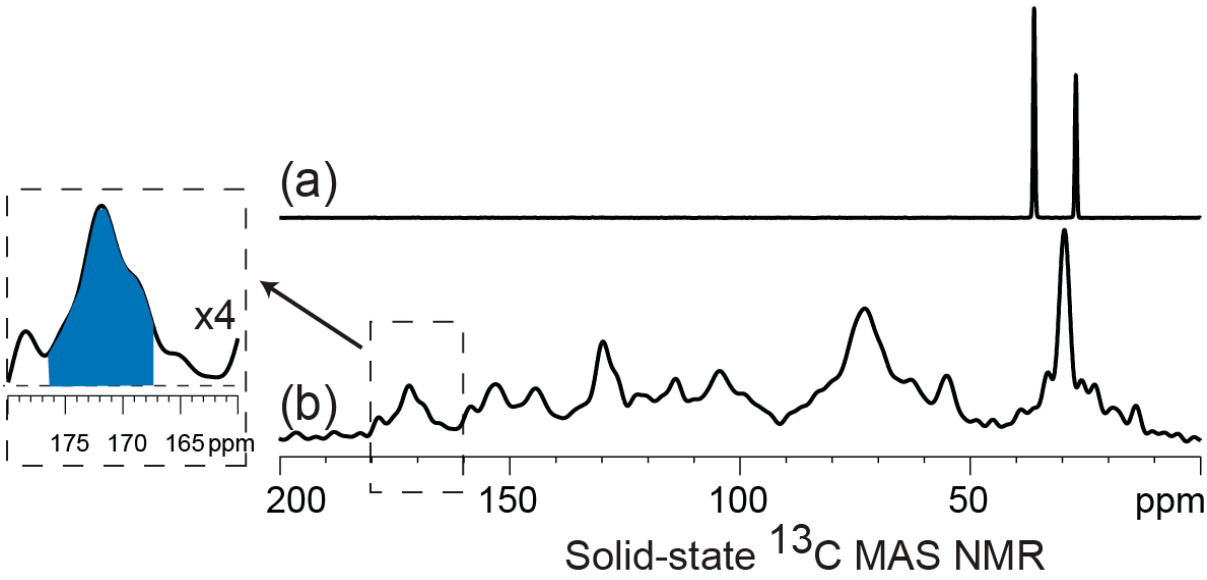
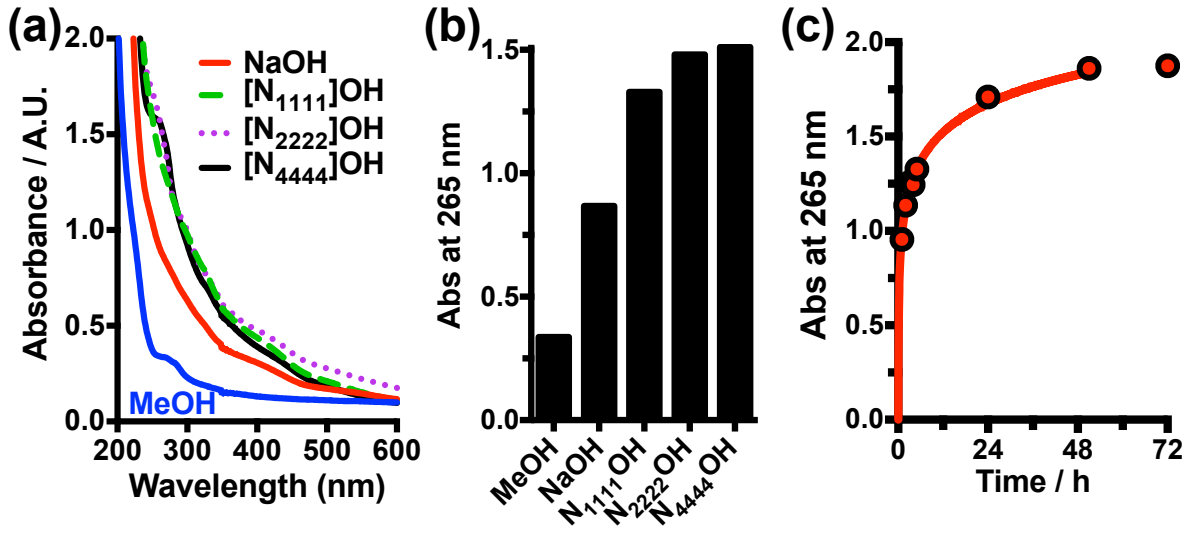


Figure 3. *Displays (a) the UV-Vis spectra recorded in a 1 mm cuvette after stirring 0.25 wt % star anise in the various solutions at room temperature for 18 h, and (b) the absorbance value from (a) at 265 nm plotted as a function of extraction media. Also shown are (c) the kinetics of extraction, plotting the absorbance at 265 nm (in a 1 mm cuvette)* vs *stirring time for 0.25 wt % star anise in* $[N_{4444}]OH \bullet 25H_2O$*; the line of best fit corresponds to* $y = 0.51 \log(x) + 0.98$.



Water Methanol NaOH $[N_{1111}]OH$ $[N_{2222}]OH$ $[N_{4444}]OH$

Figure 4. *A photograph highlighting the difference between solutions after stirring 10 wt % star anise in water, methanol and [cation]OH•25H_2O solutions at room temperature for 48 h.*